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Preparation and Characterization of Phosvitin from Hen's Egg Yolk Granule

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Soluble delipidated granule was fractionated into mainly two components by gel filtration and each component was purified by DEAE-cellulose chromatography. The phosvitins so prepared were designated as phosvitin 1 and phosvitin 2. Phosvitin 1 contained 59.2% serine and 10.2% phosphorus, while phosvitin 2 had 51.5% serine and 8.9% phosphorus. Phosvitin 1 was dissociated into nine protein fractions ranging in molecular weights from $1.33 \times 10^4 \sim 1.36 \times 10^5$ daltons, in the SDS-polyacrylamide gel electrophoretic pattern. On the other hand, Phosvitin 2 was dissociated into thirteen bands ranging in molecular weights from $1.1 \times 10^4 \sim 1.37 \times 10^5$ daltons, in the SDS-polyacrylamide gel electrophoretic pattern. On the contrary, the major component of phosvitin 1 has a molecular weight of 2.29×10^4 when determined by gel filtration with 6 M guanidine hydrochloride, while the major components of phosvitin 2 have molecular weights of 2.29×10^4 and 1.0×10^4. From these results, it seems that phosvitin is composed of several subcomponents.

The phosphorus containing protein in egg yolk has been investigated by many workers. Mecham and Olcott[1] diluted egg yolk with magnesium sulfate solution and prepared undissolved material. This fraction was non-lipid phosphoprotein and named phosvitin. It was ultracentrifugally homogeneous, but electrophoretically heterogeneous. Sugano[2] fractionated phosvitin into two components by paper electrophoresis. Sundararajan *et al.*[3] also examined phosvitin by paper electrophoresis and they obtained one rapidly moving component and two faint slower moving components. Silmot and Clagg[4] prepared a phosphoprotein from egg yolk. This was a fast moving component and electrophoretically homogeneous. On the other hand, Mok *et al.*[5] fractionated phosvitin into two components by countercurrent distribution and Connely and Taborsky[6] fractionated it into two components by ion exchange chromatography. Wallace *et al.*[7] chromatographed phosvitin into two components and they demonstrated that heterogeneity exists in these phosvitins. Clark[8] isolated phosvitin into two components by gel filtration and they demonstrated differences of chemical composition between the two components. Mano and Lipmann[9] prepared phosphoproteins with increasing levels of phosphate attached to a uniform protein. In this paper, we report the fractionation of phosvitin into constituents and on the electrophoretic behavior of the fractionated components using polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Procedure of purification. Egg yolk was diluted with an equal weight of 0.16 M NaCl, stirred and centrifuged at 4°C and 45,000 G for 30 min. The precipitated fraction was homogenized with 10 volumes of 0.16 M NaCl for 24 hr and centrifuged at 4°C and 45,000 G for 30 min. This procedure was repeated twice and the final precipitate was called granule. The granule was delipidated with 20 volumes of chloroform–methanol (2:1) and this procedure was repeated twice as has been previously reported.[10,11] The delipidated granule was called whole delipidated granule. A 5% NaCl soluble delipidated granule (soluble delipidated granule) was

then prepared from whole delipidated granule. This soluble delipidated granule was dialyzed against distilled water at 4°C and lyophilized. The soluble delipidated granule (100 mg) was applied to a column of Sephadex G-75 (2.6 × 70 cm) and eluted with 5% sodium chloride–0.01 M Tris HCl, pH 8.0 at 4°C. The eluate was collected in 5.0 ml fractions and monitored at 280 nm. The main fraction eluted was subjected to ion exchange chromatography on DEAE-cellulose using an NaCl gradient at 4 C. The eluate was monitored at 280 nm, dialyzed against distilled water and lyophilized.

Disc electrophoresis. Disc electrophoresis was performed by the procedure of Davis[12] by using 7% polyacrylamide gel at a current of 5 mA per tube. The gel was stained for protein with Coomassie brilliant blue R250 and for phosphate by the method of Cutting and Roth.[13]

Isoelectric focusing. Isoelectric focusing was performed using the procedure of Matsuo and Horio[14] with a 110 ml column at a constant voltage of 800 V for 24 hr. at 4 C. Two ml fractions were collected. The eluate was monitored at 280 nm and the pH of each fraction was measured.

SDS-polyacrylamide gel electrophoresis. SDS–polyacrylamide gel elctrophoresis was performed using the procedure of Hayashi and Ooba[15] with 10% polyacrylamide gel at a current of 8 mA per tube. The gel was stained with Coomassie brilliant blue R 250. The destained gel was estimated at an absorbance of 590 nm using an Atage Densitomaster Kemic.

Phosphate analysis. Phosphate analysis was performed by the method of Gomori.[16]

Gel filtration. Gel filtration was carried out using Sepharose 4B (colum size: 2.6 × 57 cm). The gels were equilibrated with 6 M guanidine hydrochloride and the eluate was monitored at 280 nm.

Carbohydrate analysis. Hexose was determined by the phenolsulfuric acid reaction of Dubois *et al.*[17] Hexosamine was determined by the method of Cessi and Piliego[18] using glucosamine as a standared. Sialic acid was determined by the thiobarbituric acid assay of Warren.[19]

Amino acid analysis. In the amino acid analysis of phosvitin, the procedure of Moore and Stein[20] was followed. Samples of 5 mg each were hydrolyzed in a vacuum with 1 ml of 6 N hydrochloric acid at 110 C for 24 hr. Amino acid analysis was carried out a Hitachi 835 automatic amino acid analyzer.

RESULTS AND DISCUSSION

A typical elution pattern for the soluble delipidated granule is shown in Fig. 1. The soluble delipidated granule was fractionated into four components. Both fraction 1 and fraction 2 were separately subjected to ion exchange chromatography on DEAE-cellulose. Chromatography of fraction 1 and fraction 2 on a column eluted by the gradient technique resulted in the patterns shown in Figs. 2 and 3. According to Connelly and Taborsky,[6] the maximum adsorption of phosvitin occurred at an ionic strength of about 0.1 M NaCl. As the ionic strength of the buffer was increased, protein was released. In our result, fraction 1 was eluted at a concentration of 0.30 M NaCl and fraction 2 was eluted at a concentration of 0.32 M NaCl. this result was somewhat different from the result of Connelly and Taborsky.[6] The purified fraction 1 was designated as phosvitin 1 and the purified fraction 2 as phosvitin 2. Abe *et al.*[21] fractionated phosvitin using DEAE Sepharose CL-6B at three different conditions and clarified that phosvitin was only partially frac-

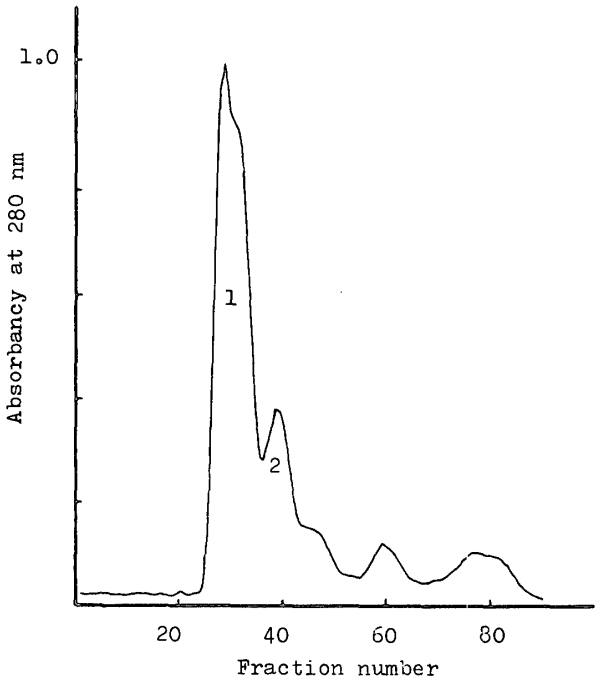

FIG. 1. Elution Profiles of Soluble Delipidated Granule on Sephadex G-75.

The column dimensions were 2.6 × 70 cm and 5 ml fractions were collected. A sample (100 mg) was applied to the column, which was equilibrated with 5% solium chloride–0.01 M Tris HCl, pH 8.0, and washed with the same buffer.

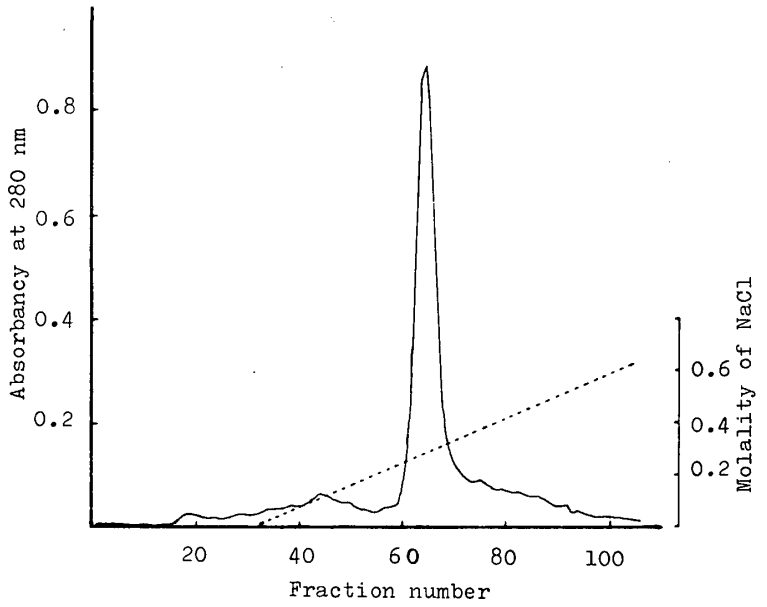

FIG. 2. Chromatography of Phosvitin 1 on DEAE-Cellulose.

The column dimensions were 2.64 × 40 cm and 10 ml fractions were collected. A sample (350 mg) was applied to the column, which was equilibrated with 0.05 M Tris HCl, pH 8.0. the solid line indicates the absorbancy of the eluate at 280 nm, and the broken line indicates the molar concentration of sodium chloride in the eluting buffer.

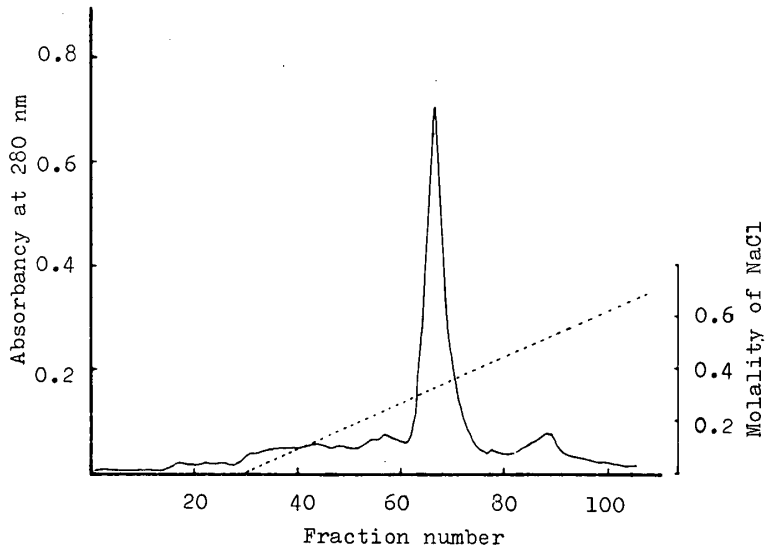

FIG. 3. Chromatography of Phosvitin 2 on DEAE-Cellulose.

The column dimensions were 2.64 × 40 cm and 10 ml fractions were collected. A sample (345 mg) was applied to the column, which was equilibrated with 0.05 M Tris-HCl, pH 8.0. The solid line indicates the absorbancy of the eluate at 280 nm, and the broken line indicates the molar concentration of sodium chloride in the eluting buffer.

tionated by ion exchange chromatography. We evaluated phosvitin 1 and phosvitin 2 by disc electrophoresis, but both proteins after electrophoresis were not stained with Coomassie brilliant blue R 250, so we stained the phosphate moiety of phosvitin. In this method, both phosvitin 1 and phosvitin 2 were resolved into three bands as shown in Fig. 4. The *Rf* value of the main component of phosvitin 1 was somewhat different from that of phosvitin 2. Isoelectrophoretic patterns of phosvitin 1 and phosvitin 2 are shown in Figs. 5 and

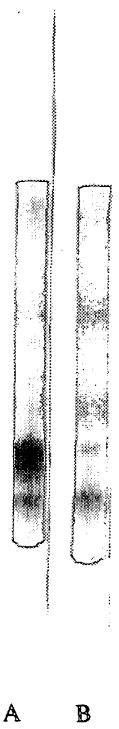

FIG. 4. Disc Gel Electrophoresis of Phosvitin 1 and Phosvitin 2.

A, phosvitin 1; B, phosvitin 2.

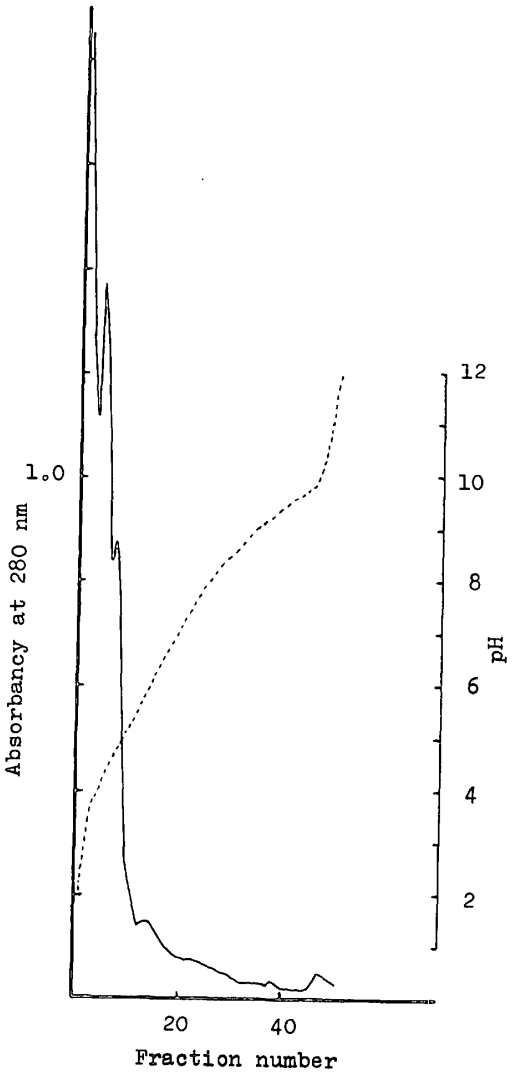

FIG. 5. Isoelectric Focusing of Phosvitin 1.

The column volume was 110 and 2 ml fractions were collected. A sample (10 mg) was applied to the column. The solid line indicates the absorbancy of the eluate, and the broken line indicates the pH of the eluate.

6. Phosvitin 1 was fractionated into three components and their isoelectric points were 2.03, 3.92 and 4.53. On the other hand, phosvitin 2 was fractionated into two components and their isoelectir points were 1.90 and 4.35. It has been reported that phosvitin contains more phosphate radicals, so phosvitin behaved as if it was an acidic polypeptide.[22~25] It seemed that phosvitin 1 and phosvitin 2 were slightly different in their chemical compositions. The electropherogram obtained from SDS–polyacrylamide gel electrophoresis is shown in Fig. 7. Schanz and Dawson[26] observed eighteen individual phosvitin bands by SDS–polyacrylamide gel electrophoresis. On the contrary, Abe *et al.*[21] analyzed phosvitin by SDS–polyacrylamide gel electrophoresis and they clarified that whole phosvitin was resolved into several components having an estimated molecular weight ranging from $6.0 \times 10^4 \sim 3.6 \times 10^4$, with the main component of β-phosvitin having a molecular weight of 4.5×10^4. On the other hand, the main components of α-phosvitin had molecular weights of 3.75×10^4, 4.25×10^4 and 4.5×10^4. Our phosvitin 1, however, was resolved into nine bands having estimated molecular weights from $1.33 \times 10^4 \sim 1.36 \times 10^5$ daltons, with the main components having molecular weights of 2.75×10^4, 3.42×10^4 and 3.82×10^4 daltons. In this way, the molecular weight of the main component of our phosvitin was somewhat different from that of Abe *et al.*[21] Macham and Olcott[1] estimated the molecular weight of phosvitin by ultracentrifugal analysis and the molecular weight found was 3.9×10^4. On the other hand, Taborsky and Mok[27] calculated the molecular weights of the major and minor

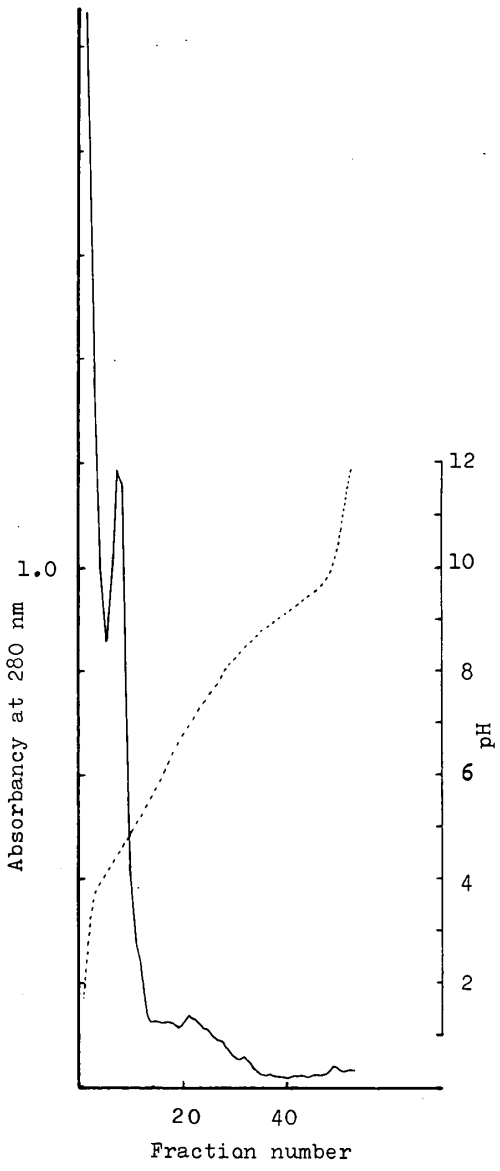

FIG. 6. Isoelectric Focusing of Phosvitin 2.

The column volume was 110 and 2 ml fractions were collected. A sample (10 mg) was applied to the column. The solid line indicates the absorbancy of the eluate, and the broken line indicates its pH.

phosvitin as 3.6×10^4 and 4.0×10^4 respectively. Clark[8] isolated phosvitin into two components by gel filtration and the major component had a molecular weight of about 3.4×10^4, while the minor one had a moleclar weight of about 2.8×10^4. In our result, fraction 1 was resolved into three components by gel filtration with 6 M guanidine hydrochloride as shown in Fig. 8, the major component having a molecular weight of 2.29×10^4, while the minor one had a molecular weight of 1.82×10^4 and 1.0×10^4. Fraction 2, however, was resolved into four components as shown in Fig. 9, the major components having a molecular weight of 1.82×10^4 and 1.0×10^4, while the minor one had a molecular weight of 2.29×10^4 and 1.48×10^4. From these results, it seems that phosvitin is composed of several subcomponents, and it was confirmed that phosvitin polypeptide makes an aggregation in the SDS solution. The phosphate content of phosvitin 1 and phosvitin 2 was 10.2 and 8.9%, respectively. Allerton and Perlmann[28] reported that the presence of non-phosphorylated protein would lead to a low result of phosphorus, and they clarified that phosvitin contained 10.4% phosphorus. This value almost agreed with that of Mok *et al.*[5] On the other hand, Abe *et al.*[21] prepared phosvitin by two methods and the phosphate content of β-phosvitin was 9.20%, while the phosphate content of α-phosvitin was 2.97%. Our phosvitin 1 contained almost the same amount of phosphorus as those reported by Allerton and Perlmann[28] and Mok *et al.*[5] The carbohydrate composition of phosvitin 1 and phosvitin 2 is shown in Table I. Phosvitin 1 contained more hexose, hexosamine and sialic acid than phosvitin 2. Allerton and Perlmann[28] have reported that phosvitin contained approximately 2.5%, by weight, of hexoses and 1.4% of glucosamine. The hexose and hexosamine contents of our phosvitin were lower than those of Allerton and Perlmann.[28] The sialic acid content of phosvitin has not previously been clarified, and it was found that phosvitin 1 contained 1.05% of sialic acid and that of phosvitin 2 was 0.64%. The amino acid compositions of phosvitin 1 and phosvitin 2 are given in Table II, the composition of phosvitin 1 being different from that of phosvitin 2. The serine content of phosvitin 1 was 59.2% and that of phosvitin 2 was 51.5%. Allerton and Perlmann[28] analyzed the amino acid composition of phosvitin and they have reported that phosvitin contained 30.0% of serine and no cysteine. Mok *et al.*[5] prepared phosvitin from the serum of estrogenized laying hens and they have reported that phosvitin contained 31.0% of serine and 0.58%

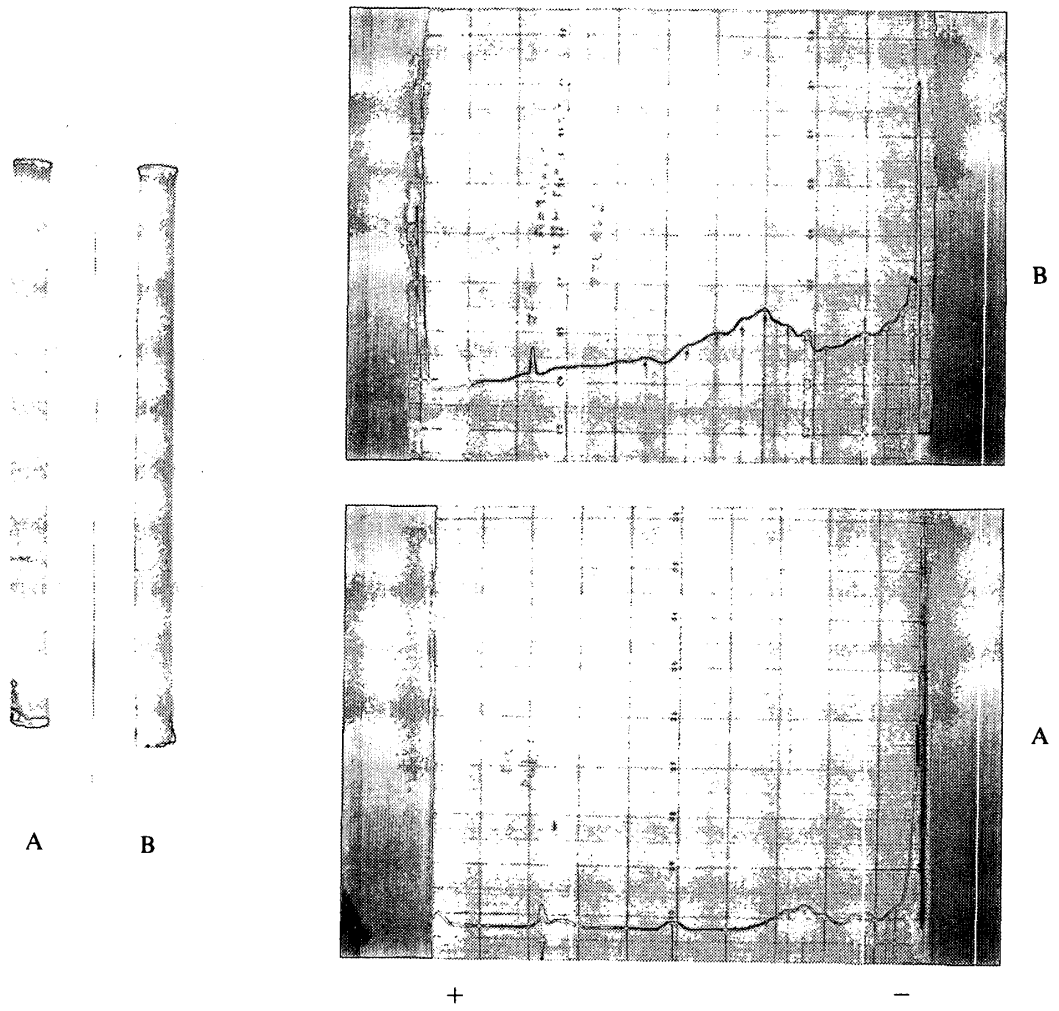

FIG. 7. SDS Polyacrylamide Gel Electrophoresis and Densitography of Phosvitin 1 and Phosvitin 2.
A, phosvitin 1; B, phosvitin 2.

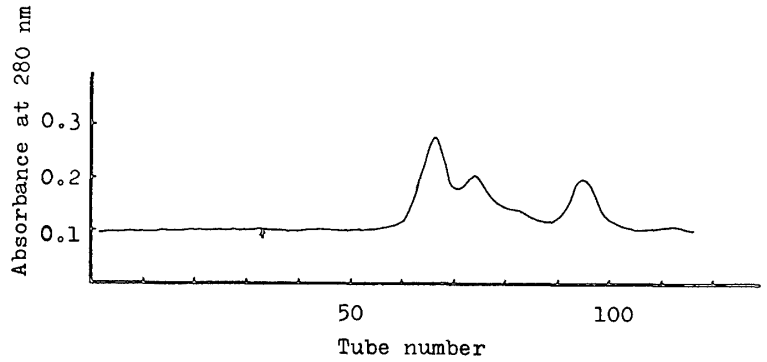

FIG. 8. Gel Filtration Pattern of Phosvitin 1 on Sepharose 4B.
Column, 2.6 × 57 cm; flow rate, 14 ml/hr; 3.5 ml fractions were collected. Solvent, 6 M guanidine hydrochloride.

of tryptophane. On the other hand, Clark[8] has reported that the serine content of phosvitin was 54.0%. It was confirmed that our phosvitin 1 contained more serine than the phosvitins reported by other researchers. Mano and Lipmann[9] have reported that phosvitin lacks sulfur containing amino acids and contains small amounts of aromatic amino acids and

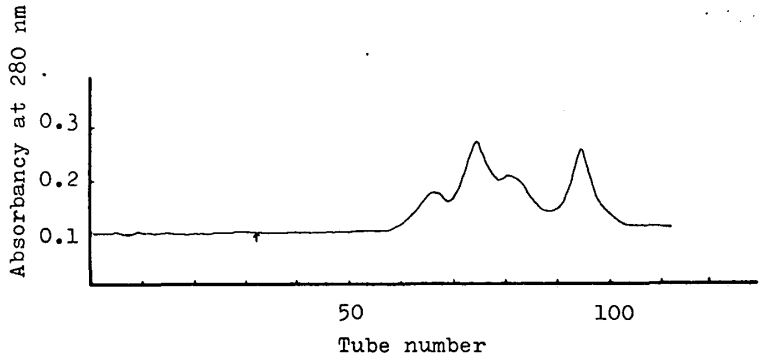

FIG. 9. Gel Filtration Pattern of Phosvitin 2 on Sepharose 4B.
Column, 2.6×57 cm; flow rate, 14 ml/hr; 3.5 ml fractions were collected. Solvent, 6 M guanidine hydrochloride.

TABLE I. CARBOHYDRATE COMPOSITION OF PHOSVITIN 1 AND PHOSVITIN 2

Fraction	Hexose (%)	Hexosamine (%)	Sialic acid (%)
Phosvitin 1	1.98	1.08	1.05
Phosvitin 2	1.46	0.73	0.64

TABLE II. AMINO ACID COMPOSITION OF PHOSVITIN 1 AND PHOSVITIN 2

	Phosvitin 1 (mol%)	Phosvitin 2 (mol%)
Asp	6.1	8.1
Thr	1.9	4.7
Ser	59.2	51.5
Glu	3.0	6.4
Pro	1.5	1.8
Gly	3.3	4.4
Ala	2.5	4.3
1/2 Cys	0.3	0.3
Val	1.1	0.9
Met	—	0.1
Ile	0.4	0.6
Leu	1.4	0.7
Tyr	—	0.3
Phe	0.4	0.6
Lys	7.6	6.0
His	5.7	3.1
Arg	5.8	6.0

histidine. Phosvitin 1 contained no methionine and tyrosine, but both phosvitin 1 and phosvitin 2 contained 0.3% cystine. From these results, it was confirmed that phosvitin 1 and phosvitin 2 were composed of several subcomponents and phosvitin 1 possesed a large quantity of serine and phosphorus containing protein.

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